

# Effects of ghrelin on protein expression of antioxidative enzymes and iNOS in the rat liver

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## Abstract

**Introduction:** We investigated the effects of ghrelin on protein expression of the liver antioxidant enzymes superoxide dismutases (SODs), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), nuclear factor  $\kappa$ B (NF $\kappa$ B) and inducible nitric oxide synthase (iNOS). Furthermore, we aimed to investigate whether extracellular regulated protein kinase (ERK1/2) and protein kinase B (Akt) are involved in ghrelin-regulated liver antioxidant enzymes and iNOS protein expression.

**Material and methods:** Male Wistar rats were treated with ghrelin (0.3 nmol/5  $\mu$ l) injected into the lateral cerebral ventricle every 24 h for 5 days, and 2 h after the last treatment the animals were sacrificed and the liver excised. The Western blot method was used to determine expression of antioxidant enzymes, iNOS, phosphorylation of Akt, ERK1/2 and nuclear factor  $\kappa$ B (NF $\kappa$ B) subunits 50 and 65.

**Results:** There was significantly higher protein expression of CuZnSOD ( $p < 0.001$ ), MnSOD ( $p < 0.001$ ), CAT ( $p < 0.001$ ), GPx, ( $p < 0.001$ ), and GR ( $p < 0.01$ ) in the liver isolated from ghrelin-treated animals compared with control animals. In contrast, ghrelin significantly ( $p < 0.01$ ) reduced protein expression of iNOS. In addition, phosphorylation of NF $\kappa$ B subunits p65 and p50 was significantly ( $p < 0.001$  for p65;  $p < 0.05$  for p50) reduced by ghrelin when compared with controls. Phosphorylation of ERK1/2 and of Akt was significantly higher in ghrelin-treated than in control animals ( $p < 0.05$  for ERK1/2;  $p < 0.01$  for Akt).

**Conclusions:** The results show that activation of Akt and ERK1/2 is involved in ghrelin-mediated regulation of protein expression of antioxidant enzymes and iNOS in the rat liver.

**Key words:** Akt, ERK1/2, superoxide dismutase, catalase, oxidative stress, nuclear factor  $\kappa$ B.

## Introduction

Ghrelin, a 28 amino acid peptide, is the main endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR) [1, 2], and is secreted primarily from the stomach, but its transcripts have also been found in the liver, suggesting its extra-endocrine as well as endocrine action [1–3]. The relationship between oxidative stress (OxS) and the expression or function of ghrelin has been reported by several research-

ers [4–6]. In addition, ghrelin administration is expected to reduce OxS and thereafter prevent the onset of different diseases [5, 7]. Moreover, ghrelin has an anti-inflammatory action on the oxidative injury of diverse organs, such as the heart, liver and pancreas [5, 8–12].

Oxidative stress reflects an excessive bioavailability of reactive oxygen species (ROS), which is the net result of an existing imbalance between production and destruction of ROS, with the latter being affected by antioxidant defenses [13]. The removal of ROS and free radicals [14] is achieved through enzymatic and non-enzymatic reactions. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) represent protective response against oxidative tissue damage [1, 15].

Inducible nitric oxide synthase (iNOS; NOS2) is a protein that produces high amounts of nitric oxide (NO), and NO is highly reactive with other free radicals. Nitric oxide reacts with superoxide ( $O_2^-$ ) producing peroxynitrite, which in turn leads to protein nitration, DNA damage, and poly (ADP-ribose) polymerase activation [16, 17]. Therefore, the predominant role of iNOS is thought to relate to OxS-mediated host defense against tumor cells and microorganisms [16, 18].

Upregulation of iNOS gene transcription requires the transcriptional factor nuclear factor- $\kappa$ B (NF $\kappa$ B) in human and rat hepatocytes [19]. In inflamed liver, liver cells are situated in an environment where NO is generated from surrounding cells and from liver cells themselves [19, 20]. It has been shown that redox-sensitive transcriptional factor NF $\kappa$ B is an important stress sensor playing a crucial role in determining cellular fate during OxS [21, 22]. Nuclear factor  $\kappa$ B is known to be activated by ROS and its activation leads to the transcriptional activation of numerous genes including antioxidant enzymes and iNOS [22–27].

The effects of ghrelin are mediated via a complex set of intracellular signaling pathways, including two kinases, extracellular regulated protein kinase (ERK1/2) and protein kinase B (Akt) [20, 28].

With the studies presented in this paper, the overall hypothesis that ghrelin is involved in antioxidative enzymes and iNOS protein expression in the liver is pursued. Furthermore, we hypothesized that ERK1/2 and Akt are involved in ghrelin-regulated antioxidant enzymes and iNOS in the rat liver. Given the complexity of the ghrelin system itself, and the limited amount of data related to the effects of ghrelin on regulation of liver antioxidant enzymes and iNOS *in vivo*, we investigated whether 5 days treatment with ghrelin caused changes in the liver protein expression of antioxidant

enzymes and iNOS, and whether these changes in their level could be related to changes of the transcriptional regulator NF $\kappa$ B as well as changes of activation of ERK1/2 and Akt.

## Material and methods

### Materials

Pentobarbital anesthetic was obtained from United Pharmaceutical Works, Prague, Czech Republic, and ether was obtained from “Lek”, Ljubljana, Slovenia. Ghrelin was obtained from Global Peptide Services, LLC Ft. Collins, CO, USA. A protease inhibitor cocktail (Complete Mini) was obtained from Roche, Mannheim, Germany. Reagents for the bicinchoninic acid (BCA) assay were purchased from Pierce (Rockford, IL, USA). Anti-actin monoclonal antibody was obtained from Sigma Aldrich Corporation (St. Louis, MO, USA) and from Abcam (Cambridge, U.K.). Anti-phospho-Akt Ser<sup>473</sup>, the anti-total Akt, the anti-phospho-ERK1/2, and the anti-total ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA), while the anti-iNOS, the anti-NF- $\kappa$ B p65 and p50 antibodies and the secondary anti-rabbit antibodies conjugated to alkaline phosphatase (ALP) or to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and from Amersham (Piscataway, NJ). Anti-MnSOD, anti-CuZnSOD, anti-GPx, and anti-GR antibodies were purchased from Stressgen (Now: Enzo Life Sciences, NY, USA) and an anti-catalase antibody from Calbiochem (CA, USA).

An enhanced chemiluminescence (ECL) kit and HRP-linked rabbit IgG were obtained from Pierce (Rockford, IL, USA) or from Amersham Pharmacia Biotech (Buckinghamshire, UK). X-ray films were purchased from Agfa (Belgium). A commercial radioimmunoassay (RIA) kit was obtained from INEP, Zemun, Serbia, and the GLUC-PAP commercial test was obtained from Randox (Crumlin, UK).

### Animals

Adult male Wistar rats (200–250 g), bred at the Institute of Biomedical Research “Galenika” in Belgrade, were used. The rats were kept in individual metabolic cages under a 12 : 12-h light–dark cycle, at 22  $\pm$  2°C, and were fed with a balanced diet for laboratory rats composed of 20% protein, 8% cellulose, 13% moisture, 1% calcium, 0.90% lysine, 0.75% methionine + cysteine, 0.5% phosphorus, 0.15–0.25% sodium, vitamin mixture (A 10,000 IU/kg, D 3 1,600 IU/kg, E 25 mg/kg, B 12 0.02 mg/kg), a mixture of minerals (in milligrams per kg: zinc 100, iron 100, manganese 30, copper 20, iodine 0.5, selenium 0.1), antioxidants 100 mg/kg, and digestible/metabolizable energy 11 MJ/kg (prepared by “D. D. Veterinarski zavod

Subotica", Subotica, Serbia). Food and water were available to rats *ad libitum*. Body weight was measured daily.

Experimental protocols were approved by the official Vinca Institute's Ethical Committee for Experimental Animals and conformed to the recommendations given in "Guide for the Care and Use of Laboratory Animals" (1996 National Academy Press, Washington, D.C., USA).

### Surgical procedures

Surgical procedures were performed under pentobarbital (45–50 mg/kg; intraperitoneally) and ether anesthesia. The rats were implanted with a headset and later used for intracerebroventricular (ICV) injections. A minimum recovery time of 5 days was permitted before the onset of experiments. The headset consisted of a silastic-sealed 20G cannula [20, 29–31], implanted into a lateral cerebral ventricle 1 mm posterior and 1.5 mm lateral to the bregma and 3 mm below the cortical surface. A small stainless steel anchor screw was placed at a remote site on the skull. The cannula and screw were cemented to the skull with dental acrylic.

### Experimental treatment

After recovery, rats were divided into two groups (each consisting of six animals). The first group of rats, labeled as ghrelin, was treated ICV with 1 µg of ghrelin dissolved in 5 µl of phosphate buffered saline (PBS) every 24 h for 5 consecutive days [20, 30, 31]. The second group of control rats, labeled as CONTROL, was injected with 5 µl of PBS. All ICV treatments were administered between 10:00 and 11:00 a.m., and animals were euthanized by decapitation under deep ether anesthesia 2 h after the last ICV injection. Livers were excised, weighed, and used for experiments. Blood was collected from each rat immediately after decapitation. Serum samples were stored at –20°C for measuring insulin, glucose and free fatty acid (FFA).

### Liver lysate preparation

After decapitation, rat livers were homogenized on ice with an Ultra-turrax homogenizer in buffer (pH 7.4) containing (in mM): 150 NaCl, 50 Tris, 2 EDTA, 10% glycerol, 1% Triton X-100, protease inhibitor cocktail with additional 2 mM PMSF, and 2 mM sodium orthovanadate. Homogenates were centrifuged at 600×g for 20 min at 4°C, and obtained supernatants were ultra-centrifuged for 60 min at 100,000×g [32], and used as the cytoplasmic fraction. Protein concentration was determined by the BCA method using bovine serum albumin as standard.

### Determination of serum insulin, glucose and free fatty acid concentrations

Serum insulin levels were determined by the RIA method, using rat insulin standards, with assay sensitivity of 0.6 mIU/l and an intra-assay coefficient of variation of 5.24% and expressed as mIU/l of serum. The glucose levels were measured using a GLUC-PAP kit according to the manufacturer's manual and expressed as mmol/l. For measurement of serum insulin, glucose and free fatty acid (FFA) levels animals were fasted overnight before collection of blood samples in order to avoid changes of insulin, glucose and FFA level induced by food intake. The serum FFA levels were determined using a modified version of Duncombe's colorimetric method [33] and expressed as mmol/l.

Also, fasting serum concentrations of both glucose and insulin were used to calculate the indices of insulin resistance [homeostasis model assessment (HOMA)-IR] and insulin secretion (HOMA-β) with the following formulae [34]:  $\text{HOMA-IR} = \text{fasting insulin } (\mu\text{U/ml}) \times \text{fasting glucose (mM)} / 22.5$  and  $\text{HOMA-}\beta = 20 \times \text{fasting insulin } (\mu\text{U/ml}) / (\text{fasting glucose (mM)} - 3.5)$ .

### SDS-PAGE and Western blotting

Equal amounts of protein (50 or 60 µg/lane) were separated by 10% SDS polyacrylamide gels [35–37] and transferred to polyvinylidene difluoride membranes as previously described [38, 39]. The membranes were blocked with either 5% nonfat dry milk or 5% bovine albumin and probed with antibodies against MnSOD (in a dilution of 1 : 4000), CuZnSOD (in a dilution of 1 : 10000), GPx (in a dilution of 1 : 1000), GR (in a dilution of 1 : 3000), iNOS (in a dilution of 1 : 2000), NFκB subunits p50 (in a dilution of 1 : 1000) and p65 (in a dilution of 1 : 500). After washing, membranes were incubated with the appropriate secondary ALP-conjugated anti-rabbit antibody (in a dilution of 1 : 2000) and used for subsequent detection with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium, or with the appropriate secondary HRP-conjugated anti-rabbit antibody (in a dilution of 1 : 2000) or with ECL Rabbit IgG, HRP-linked whole antibody (in a dilution of 1 : 5000) or with ECL Mouse IgG, HRP-linked whole antibody (in a dilution of 1 : 2000) and used for subsequent detection by using an ECL kit. After analysis of pAkt (in a dilution of 1 : 1000) and pERK1/2 (in a dilution of 1 : 1000), membranes were stripped and reblotted with antibody detecting total Akt (in a dilution of 1 : 1000) or total ERK1/2 (in a dilution of 1 : 1000). In order to ensure equal protein loading, all blots were probed with mouse anti-β-actin monoclonal antibody. Densitometry of protein bands on X-ray film was performed by Image J analysis PC software.

## Statistical analysis

Values are expressed as means  $\pm$  SEM, with  $n$  values representing the number of experiments. Statistical significance was evaluated with Student's  $t$  test. The SPSS program for Windows (SPSS, Chicago, IL, USA) was used for statistical analyses. Value of  $p < 0.05$  was considered significant.

## Results

### Body mass, plasma and serum metabolic profile

Initial body mass was comparable in both the control and ghrelin groups. Mass gain during the 5 days of ghrelin treatment was not changed significantly. Blood glucose levels did not change in the ghrelin group compared with the control group. Plasma insulin level was significantly increased in ghrelin-treated rats ( $p < 0.01$ ). Due to hyperinsulinemia and a slight non-significant increase in glucose level, HOMA-IR and HOMA- $\beta$  indices were significantly ( $p < 0.05$ ) higher in the ghrelin group. Plasma FFA was significantly ( $p < 0.01$ ) lower in the ghrelin than in the control group (Table I).

### Effects of ghrelin on protein expressions of CuZnSOD, MnSOD, CAT, GPx and GR in rat liver

We designed experiments to elucidate the possible antioxidant properties of ghrelin by measuring protein expression of the major antioxidant enzymes such as SOD, CAT, GPx, and GR in the rat liver following 5 days of administration of ghrelin. Protein expression of cytoplasmic CuZnSOD, MnSOD, CAT (Figure 1), GPx and GR (Figure 2) was significantly higher ( $p < 0.05$ ;  $p < 0.05$ ;  $p < 0.01$ ,  $p < 0.001$ , respectively) in ghrelin-treated, compared with control rats. Taken together, those results indicate that ghrelin increased protein expression of antioxidative enzymes in the rat liver.

### Effects of ghrelin on iNOS protein expression and protein level of NF $\kappa$ B in the rat liver

We have previously shown that ghrelin administration caused an increase in serum NO [20]. Because ghrelin stimulates total plasma NO pro-

duction [20], we next examined whether this effect of ghrelin is due to upregulation of iNOS. In contrast to regulation of NO plasma production [20], ghrelin significantly ( $p < 0.001$ ) reduced iNOS protein level in the liver by 42% compared with controls (Figure 3).

The promoter of the gene for iNOS is the binding site for many transcription factors including NF $\kappa$ B in the 5' region of the gene for iNOS [12, 40]. When rats were treated with ghrelin, the levels of NF $\kappa$ B subunits p65 (Figure 4 A) and p50 (Figure 4 B) were significantly lower by 30% (p65,  $p < 0.001$ ) and by 24% (p50,  $p < 0.05$ ) compared with controls, respectively. These data suggest that NF $\kappa$ B plays a significant role in ghrelin-regulated antioxidant enzymes and iNOS expression in the rat liver.

### Liver ERK1/2 and Akt phosphorylation

Since we have previously shown that stimulation of iNOS involves activation of Akt and ERK1/2 by phosphorylation in the heart tissue [20], we next examined the effect of ghrelin on phosphorylation state of Akt and ERK1/2 (Figure 5). Ghrelin significantly increased ( $p < 0.01$ ) phosphorylation of Akt at Ser<sup>473</sup> (Figure 5 B) and ERK1/2 at Tyr<sup>42</sup> and Tyr<sup>44</sup> ( $p < 0.05$ ) (Figure 5 A). Thus, the regulation of liver antioxidative enzymes and iNOS protein expression by ghrelin requires activation of both ERK1/2 and Akt.

## Discussion

The results of the present study demonstrate that ghrelin enhanced liver protein expression of ZnSOD, MnSOD, CAT, GPx and GR. Furthermore, ghrelin reduced liver protein expression of both iNOS and p65 and p50 subunits of NF $\kappa$ B. In addition, ghrelin stimulates the activity of Akt and ERK1/2 kinase.

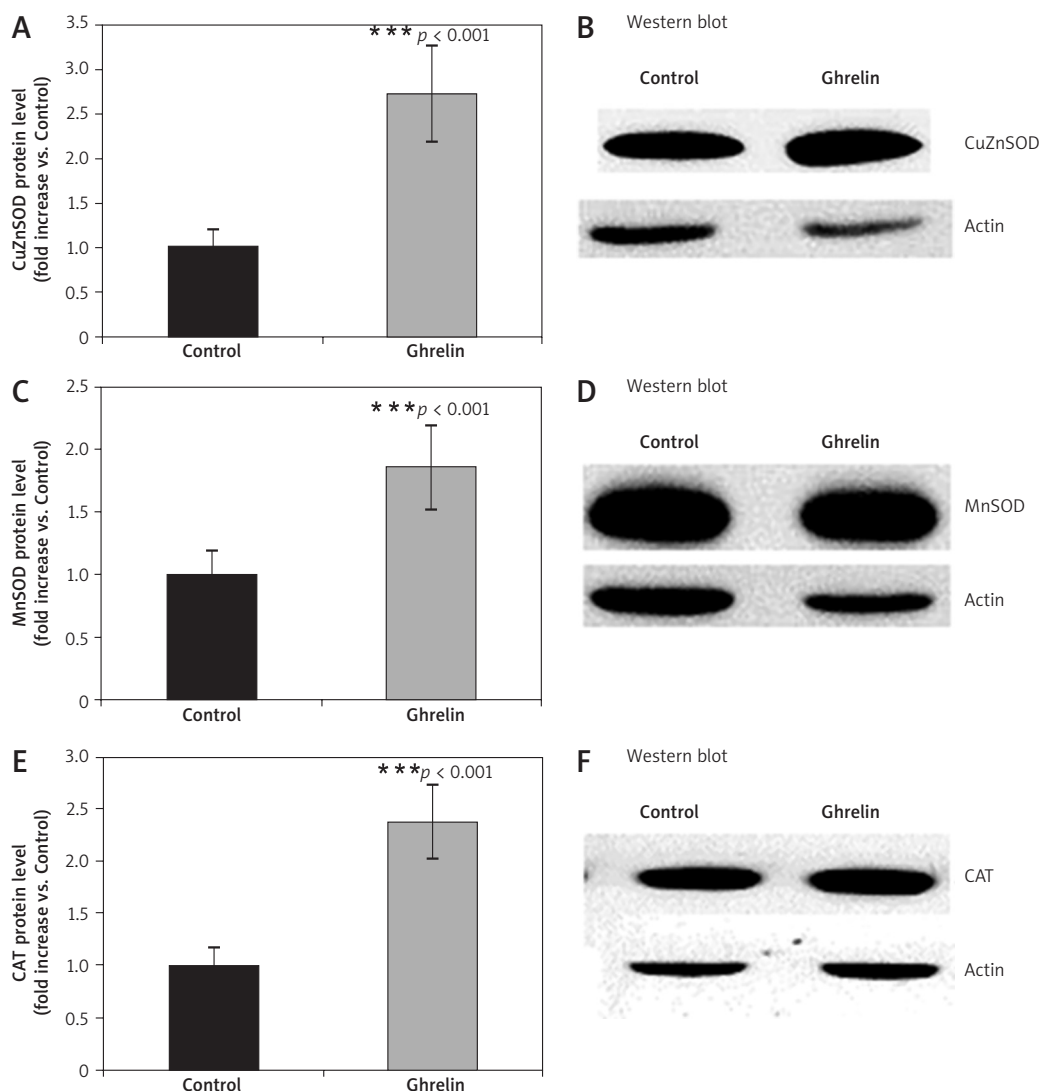
Low doses of ICV ghrelin treatment for 5 days did not increase body mass (Table I). These results are in agreement with reports by others which show that after ghrelin treatment, there were no significant increases in body mass gain [41]. Also, these results are not in agreement with literature data reported by others [31, 42–44]. This inconsistency in the results could be explained by different experimental approaches, different ways of treating animals as well as doses of applied ghrelin. In

**Table I.** Body mass and biochemical characteristics of rats in control and ghrelin treated groups. Values are given as mean  $\pm$  SEM;  $p < 0.05$  was considered significant

Experimental groups	Initial body weight [g]	Final body weight [g]	Liver weight [g]	Glucose [mmol/l]	Insulin [mIU/l]	HOMA-IR	HOMA- $\beta$	FFA [mmol/l]
Control	234 $\pm$ 8	235 $\pm$ 11	9.49 $\pm$ 0.72	8.32 $\pm$ 0.20	96 $\pm$ 7	33 $\pm$ 2	375 $\pm$ 16	0.77 $\pm$ 0.05
Ghrelin	215 $\pm$ 8	226 $\pm$ 8	8.85 $\pm$ 0.47	8.56 $\pm$ 0.06	193 $\pm$ 18**	69 $\pm$ 7*	711 $\pm$ 71*	0.49 $\pm$ 0.03**

HOMA-IR index of insulin resistance (homeostasis model assessment), HOMA- $\beta$ -index of insulin secretion, FFA – free fatty acids,

\* $p < 0.05$ ; \*\* $p < 0.01$



**Figure 1.** Effects of ghrelin on CuZnSOD (A and B), MnSOD (C and D) and CAT (E and F) protein expression in the rat liver. The results are expressed as a fold increase of control (CONTROL arbitrarily set at 1). Results are mean  $\pm$  SEM of 4 separate experiments;  $p < 0.05$  was considered significant (\*\*\* $p < 0.001$ ). B, D, and E are representative Western blots

CuZnSOD – copper/zinc dependent superoxide dismutase, MnSOD –  $Mn^{2+}$  dependent superoxide dismutase, CAT – catalase

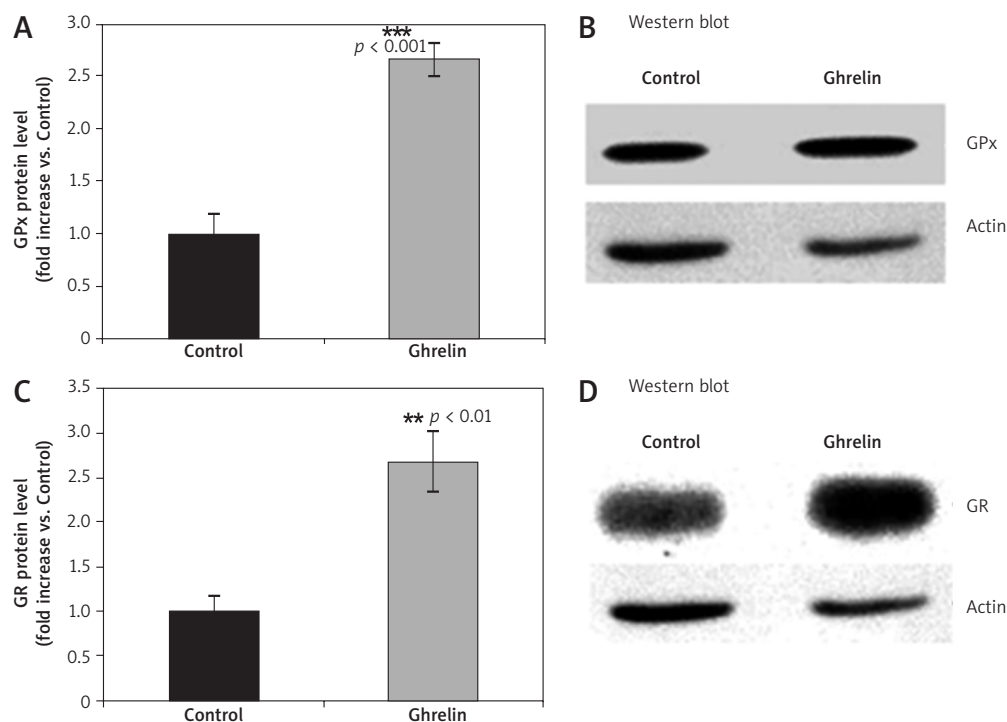
addition, it is possible that the use of different rat strains and different species of animals also contributed to the inconsistency in the results.

Treatment with ghrelin leads to an increase in serum insulin concentration, but had no effect on serum glucose concentration, which is consistent with results obtained by others [31, 44]. It is important to note that the findings in the literature of ghrelin's effect on insulin secretion are contradictory [45–47]. Lee *et al.* [48] reported that ghrelin treatment leads to stimulation of insulin secretion while Kamegai *et al.* [49] reported no significant increase in insulin concentration after ICV ghrelin treatment. It was found that intravenous administration of ghrelin inhibits insulin secretion even at increased concentrations of glucose in humans [46, 50]. The contradiction in the results concern-

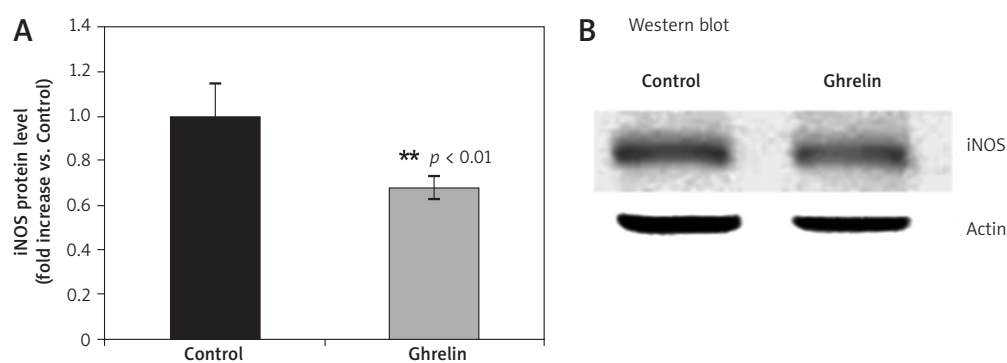
ing the effects of ghrelin on insulin levels can be explained by different doses of applied ghrelin [51]. In addition, Arvat *et al.* [52] demonstrated that ghrelin leads to the release of GH in rats and humans. Injected ICV ghrelin also leads to stimulation of GH release in rats [41], with much lower doses of ghrelin having a more significant effect on the secretion of this hormone [53]. It is considered that the effects of ghrelin on insulin level were achieved via a central effect, i.e. its effect on the synthesis of GH, given the low dose of ghrelin and short half-life time in circulating ghrelin [41, 54].

We used index values of HOMA-IR and HOMA- $\beta$  to determine whether ghrelin induces IR and affects the ability of pancreatic  $\beta$  cells to secrete insulin in rats. Both indices show a significant increase in ghrelin-treated compared with control





**Figure 2.** Effects of ghrelin on GPx (A and B) and GR (C and D) protein expression in the rat liver. The results are expressed as a fold increase of control (CONTROL arbitrarily set at 1). Results are mean  $\pm$  SEM of 4 separate experiments;  $p < 0.05$  was considered significant (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). B and D are representative Western blots  
GPx – glutathione peroxidase, GR – glutathione reductase

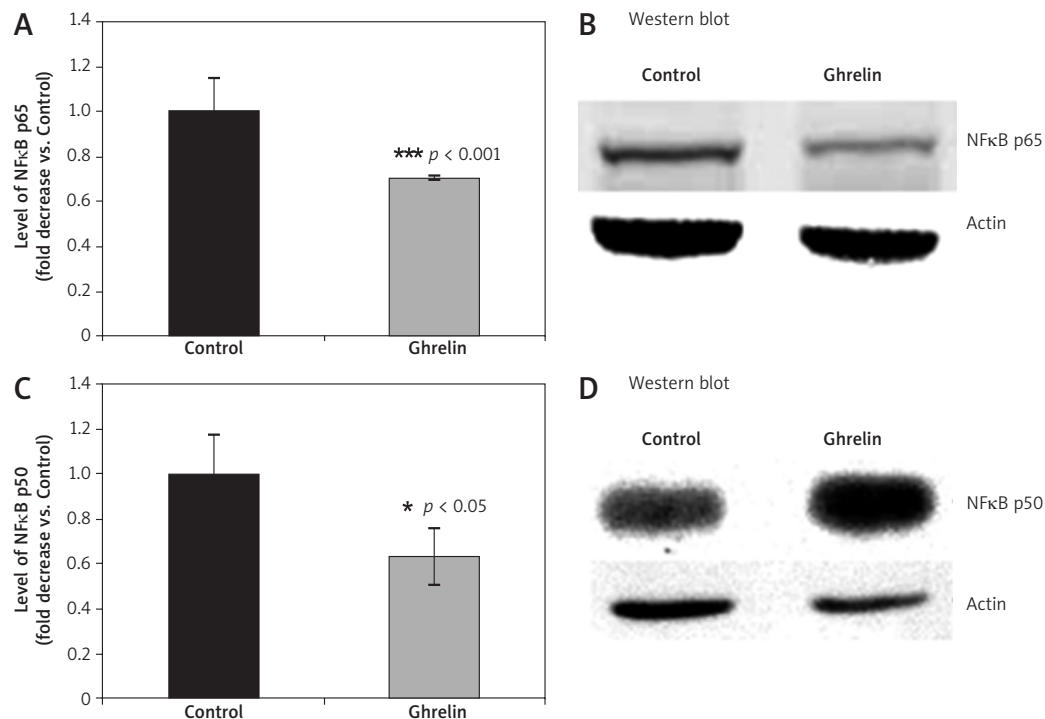


**Figure 3.** Effects of ghrelin on iNOS protein level in the rat liver. The results are expressed as a fold increase of control (CONTROL arbitrarily set at 1). Results are mean  $\pm$  SEM of 4 separate experiments;  $p < 0.05$  was considered significant (\*\* $p < 0.01$ ). B is a representative Western blot  
iNOS – inducible nitric oxide synthase

rats, suggesting that there has been development of IR and insulin secretion in treated animals. In the early stages of IR, in order to achieve the physiological values of glucose, there is a compensatory increase in insulin concentrations, as our results demonstrated. However, after a period of compensated IR there is impairment in the transport of glucose, although insulin concentrations increased with increasing IR. The results of others on the effect of ghrelin on IR are controversial [55]. Poykko *et al.* [56] found that low levels of ghrelin in the plasma lead to the development of IR, while Vestergaard *et al.* [57] reported that ghrelin stimulates the development of peripheral IR.

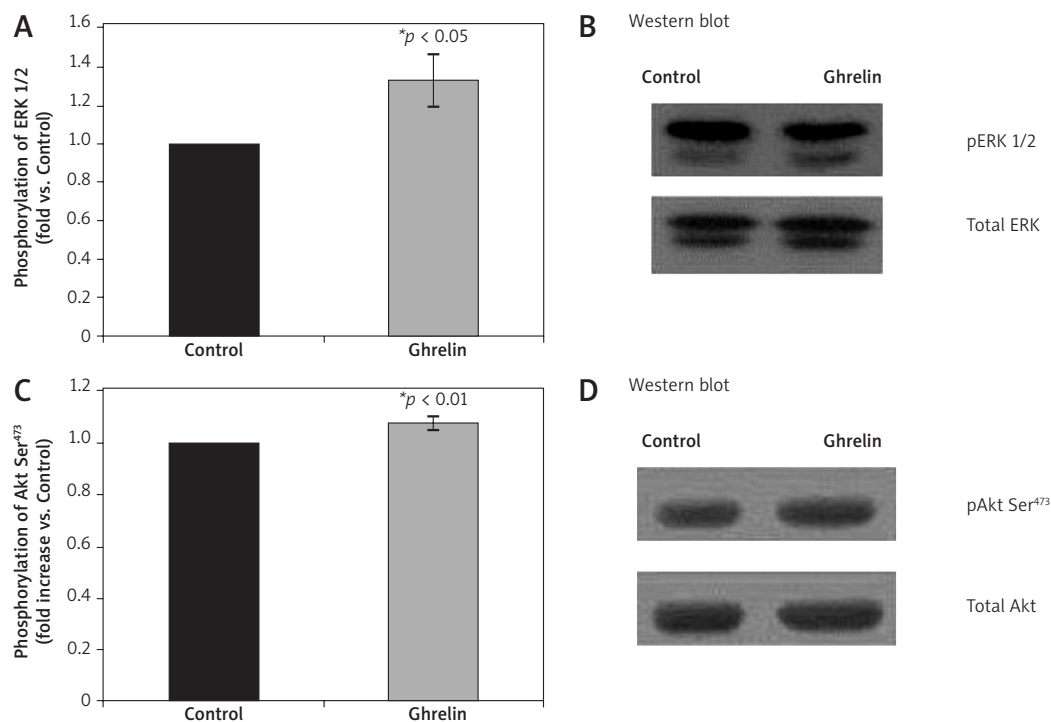
We observed that ghrelin treatment leads to a significant decrease in FFA concentration (Table I). It is possible that the levels of FFA in the serum of animals treated with ghrelin are reduced as they are transported and stored in the cells due to increased levels of circulating insulin. The FFA and glucose are transported from the circulation into muscle cells and cardiomyocytes after meals or when cells have an increased need for them [58–60].

Our data show increased protein expression of liver ZnCuSOD, MnSOD, CAT, GPx and GR by ghrelin. The ability of ghrelin to significantly increase liver protein expression of antioxidant enzymes has



**Figure 4.** Effects of ghrelin on levels of p65 (A and B) and p50 NFκB (C and D) subunits in the rat liver. Results are expressed as a fold increase of control (CONTROL arbitrarily set at 1). Results are mean  $\pm$  SEM of 4 separate experiments;  $p < 0.05$  was considered significant ( $***p < 0.001$ ). B and D are representative Western blots

NFκB – nuclear factor κB



**Figure 5.** Effects of ghrelin on ERK1/2 (A and B) and Akt (C and D) phosphorylation in the rat liver. The results are expressed as a fold increase of control (CONTROL arbitrarily set at 1). Results are mean  $\pm$  SEM of 4 separate experiments;  $p < 0.05$  was considered significant ( $*p < 0.05$ ;  $**p < 0.01$ ). B and D are representative Western blots

ERK1/2 – phospho protein kinase p44/42, Akt – protein kinase B

not been demonstrated in the rat liver *in vivo*, and its intracellular signal transduction pathways are not well understood. Our results confirm previous data on the antioxidative properties of ghrelin in other tissues [1, 6, 11, 61–63]. Several researchers reported the relationship between OxS and the expression or function of ghrelin [4–6]. Previous studies have reported that ghrelin has an anti-inflammatory action on the oxidative injury of diverse organs, such as the heart, liver and pancreas [5, 8–12]. *In vitro* studies, using human polymorphonuclear cells incubated with ghrelin, showed that ghrelin inhibited ROS generation as measured by chemiluminescence [5, 64]. Iseri *et al.* [5, 65] reported that ghrelin possesses antioxidant and anti-inflammatory characteristics. Our observation of ghrelin stimulation of liver antioxidative protein expression is consistent with previous observations obtained from *in vitro* studies, where the authors proposed that ghrelin could protect the liver against oxidative damage [1].

Our data may indicate that ghrelin's effect on protein expression of antioxidant enzymes is dependent on some other signaling pathways, such as the insulin-like growth factor-1 (IGF-1) signaling pathway. Also, these findings raise the possibility that ghrelin has indirect effects in the regulation of liver actions through IGF-1. However, administration of ghrelin did increase plasma IGF-1 [66]. Similar to our findings in the heart [20], findings from this study support the notion that ghrelin and IGF-1 may act in parallel signaling pathways, both of which are necessary for the regulation of antioxidative enzymes, and that there is a cross-talk between ghrelin and IGF-1, where ghrelin is activated first, which further results in the activation and/or induction of IGF-1. If induction or activation of IGF-1 receptors is needed for the action of ghrelin, this would explain why ghrelin induces protein and gene expression of antioxidative enzymes [20].

In this study, ghrelin reduced liver iNOS protein expression (Figure 3). In addition, using qRT-PCR analysis (data not shown), we measured the effects of ghrelin on the amount of *Nos2* mRNA and the relative rate of transcription of the *Nos2* gene. Our preliminary results indicated that changes in *Nos2* mRNA level were due to changes in regulation of its transcription. Reduced *Nos2* gene expression by ghrelin is in agreement with the literature data showing down-regulated expression of gastric iNOS by ghrelin [40]. These data extend our previous findings in the heart [20] that ghrelin regulates iNOS [20]. According to our knowledge there is a lack of literature data about *in vivo* effects of ghrelin on iNOS in rat liver.

The promoter of the gene for iNOS is the binding site for many transcription factors including NFκB in the 5' region of the gene for iNOS [40, 67].

Our results show that the levels of both p50 and p65 subunits of NFκB were decreased in rat liver cytoplasm after treatment with ghrelin. Thus, it is possible that ghrelin *in vivo* suppresses IκB-α phosphorylation and degradation, inhibits NFκB nuclear translocation, and prevents iNOS gene induction [68]. These results suggest that NFκB may represent an important regulatory factor in ghrelin-induced expression of iNOS in the rat liver.

Studies in different cell types provide evidence that NFκB activation is modulated by oxidative stress [69, 70]. Conversely, increased levels of ROS are associated with activation of NFκB [70–72]. In addition, it has been shown that increasing the ROS-inactivation capacity inside the β cell could interrupt the cytokine-induced activation of NFκB and reduce the subsequent activation of the iNOS promoter [70].

The signaling pathway of the ERK1/2 cascade of molecules triggers phosphorylation of one another, ultimately resulting in activation of NFκB and regulation of gene transcription. When activated by phosphorylation, ERK1/2 can phosphorylate many target proteins, including transcription factors and protein kinases [73]. The role of ERK1/2 in the induction of iNOS has been demonstrated in several systems [67, 74–76]. Induction of iNOS is mediated through the stimulation of ERK1/2 by NFκB [67, 76]. In addition, it has been shown that ghrelin can activate the signaling pathway IRS-1/MAPK [77]. The role of the ERK1/2 signaling network in ghrelin-induced activation of NFκB and iNOS expression was demonstrated by measuring the level of phosphorylation of ERK1/2. The results indicate that ghrelin leads to increased phosphorylation of ERK1/2 in the liver (Figure 5 A).

On the other hand, literature data clearly indicate the important role of Akt in the regulation of NFκB and iNOS activation [68]. The results of our study show that ghrelin can activate Akt in the liver (Figure 5 B). These results support the fact that activation of Akt mediates the effects on iNOS and NFκB. These results are in agreement with the results reported by others [68], showing that the effects of ghrelin on LPS-induced up-regulation of IKK-β and iNOS in gastric mucosa involves Akt activation [68]. Signaling through the Src/Akt pathway is known to occupy a central stage in the receptor (GHS-R)-mediated responses to ghrelin stimulation [68, 78, 79], and the recent reports suggest that the activity of IKK complex may also be subject to inhibition through S-nitrosylation of a specific cysteine residue within the activation loop of IKK-β by the endogenous NO donors [68, 80–82].

The data presented in this paper indicate that regulation of the rat liver oxidative stress enzymes and iNOS by ghrelin involves the Akt/



ERK1/2 pathways because both Akt and ERK1/2 phosphorylation were increased by ghrelin administration.

The limitations of this experimental work are that no pathological insult was given to the animals to increase the reactive oxygen species in liver and then prove that ghrelin is indeed a protective and anti-oxidant peptide under pathophysiological conditions. We plan to investigate the effects of ICV administered ghrelin on antioxidative enzymes as well as iNOS in liver from high fat fed rats, a model of obesity associated with insulin resistance. This will allow us to investigate the beneficial effects of ghrelin on antioxidative enzymes as well as iNOS in pathophysiological conditions, such as obesity associated with insulin resistance. We believe that the positive effects of ghrelin in the high fat model rats will provide a reasonable rationale to further investigate the molecular mechanisms involved in ghrelin's effects on oxidative stress in pathophysiological conditions.

In conclusion, administration of ghrelin could promote an antioxidative defense system in the rat liver by increasing expression of antioxidative enzymes and reducing iNOS and NF $\kappa$ B protein levels through activation of Akt and ERK1/2 kinases. This finding extends previous reports concerning the antioxidative properties of ghrelin in other tissues [1, 5, 6, 63, 65, 68, 83–86].

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